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Spread of Measles Virus Through Axonal Pathways Into Limbic Structures in the Brain of TAP1 -/- Mice

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The spread of measles virus into the brain was studied exploiting the olfactory pathway, which represents an important route of neuroinvasion by viruses. The virus was injected into the main olfactory bulb of wild-type mice and mice with disrupted TAP1 gene (TAP refers to the Transporter associated with Antigen Presentation), which codes for products essential for the cell-mediated immune response. Virus invasion was monitored for 4 weeks by immunohistochemistry. The distribution of measles virus was found to be restricted to brain areas connected with the olfactory bulbs. However, in the wild-type mice there was a marked infiltration of lymphocytes in the infected brain structures, and the virus did not pass beyond the piriform cortex. In the TAP1 -/- mice the virus spread more extensively along olfactory projections into the limbic system and monoaminergic brainstem neurons. Infected mice of both types developed seizures, which may have been focally evoked from the piriform cortex. This study provides evidence that measles virus can spread through axonal pathways in the brain. The findings obtained in the gene-manipulated mice point out that a compromised immune state of the host may potentiate targeting of virus to the limbic system through olfactory projections. ***J. Med. Virol. 52:362–369, 1997.*** © 1997 Wiley-Liss, Inc.

KEY WORDS: MHC molecules; olfactory system; axonal transport; seizures

INTRODUCTION

Spread of viruses along axonal pathways has attracted interest in studies on pathogenetic mechanisms behind targeting of viral infections in the brain. In this respect, the olfactory pathways represent an important route for neuroinvasion by viruses, because it connects olfactory neurons, which are exposed to the environment in the nasal cavity to the brain and, in particular, to limbic and brainstem monoaminergic cells [Carson, 1984; Shipley and Adamek, 1984]. In the nasal cavity certain viruses can attack preferentially the neuroepithelia, while others infect mainly the respiratory epithelium [Lundh et al., 1987; Babic et al., 1994]. A number of viruses, such as rabies, vesicular stomatitis, and herpes simplex virus, after infection of the olfactory bulbs may propagate further into the brain through axonal pathways in anterograde and/or retrograde directions to reach different nerve cell groups and regions [for review see, Mohammed et al., 1993; Tyler and Fields, 1996]. Such viral infections can lead to behavioral changes of the animals. For instance, we have previously described that a temperature-sensitive mutant of vesicular stomatitis virus injected intranasally into 12-day-old rats can reach in the brainstem serotonergic raphe neurons and cause a nonlethal infection with selective serotonin depletion in the neocortex and persistent behavioral disturbances [Lundh et al., 1988; Mohammed et al., 1990, 1991; Andersson et al., 1993].

The present study was undertaken to determine whether a common airborne virus, namely measles virus, which has not been hitherto associated with axonal transport, can spread in the brain along axonal pathways after olfactory bulb injections. The study was carried out partly on mice with genetic defect affecting the cellular immune defense, since it was assumed that such mice may show an enhanced spread of the virus within the brain. For this purpose mice were used with disrupted gene for the Transporter associated with Antigen Presentation (TAP). During a virus infection, a fraction of all viral proteins is degraded in the cytoplasm into short peptides. Peptide antigen is subsequently translocated over the endoplasmic reticulum (ER) membrane by TAP 1/2 gene products. Once in the ER, appropriate peptides bind major histocompatibility

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complex (MHC) class I molecules and this complex is then transported to the cell surface for scrutiny by cytotoxic T lymphocytes (CTL) [for review see, Yewdell and Bennink, 1992]. In gene knock-out TAP1 -/- mice, which lack a functional TAP complex [Van Kaer et al., 1992], the access of viral antigens to the ER is prevented and, as a consequence, viral antigens fail to associate with MHC class I molecules. Thus, the TAP1 -/- mice provide an excellent tool to study the effect of viral spread without interference by a normal MHC class I restricted CTL-mediated immune response [Van Kaer et al., 1992; Ljunggren and Van Kaer, 1995].

MATERIAL AND METHODS

Animals and Virus Inoculation

Twenty-nine male TAP1 -/- mice backcrossed to C57BL/6 mice [Van Kaer et al., 1992], and 33 wild-type C57BL/6 (B6) mice were used. TAP1 -/- mice were a kind gift from Dr. L. Van Kaer (Vanderbilt University, Nashville, TN) and B6 mice were obtained from Bommice, Bomholtgård, Ry, Denmark. The animals, which were 8–10-weeks-old, were inoculated with the HNT strain of measles virus, kindly supplied by Dr. K.W. Rammohan, Ohio State University, Columbus, OH. A titer of $10^{4.7}$ 50% intracerebral lethal doses/ml ($ICLD_{50}$), assayed in newborn BALB/c mice was used. Under chloral hydrate anesthesia (350 mg/kg intraperitoneally), the animals were placed in a stereotaxic frame. Following skin incision and exposure of the skull, a small hole was made above the middle part of right main olfactory bulb. One μl of viral suspension was injected at a depth of 1.5 mm below the dura, with the use of a Hamilton syringe, at the rate of 0.2 μl/min. The needle was left in situ for 3 min after the injection to minimize the leakage of the inoculum along the needle track. The skin incision was sutured with metal clips.

Histology and Immunohistochemistry

Histological and immunohistochemical analyses were carried out in brains sampled at 2 (n = 4), 4 (n = 4), 7 (n = 8), 10 (n = 11), 14 (n = 9), and 28 (n = 10) day (d) postinfection (p.i.). An equal number of wild-type and TAP -/- mice were sampled at each time point; on the 10^{th} and 14^{th} d p.i. one additional wild-type and TAP1 -/- mouse, respectively, were sampled. The animals were anesthetized with an overdose of chloral hydrate and perfused transcardially with 10 ml of 0.9% NaCl followed by 20 ml of 4% paraformaldehyde in phosphate-buffered saline, pH 7.4 (PBS). The brains were removed, postfixed in 4% paraformaldehyde in PBS at 4°C for 10–24 hr, soaked in 20% sucrose in PBS, and kept at 4°C for 36–48 hr. Coronal sections were cut at 20 μm thickness on a cryostat, and collected in four series on slides covered with 0.5% gelatin/0.25% $KCr(SO_4)_2$. An uninfected mouse brain was similarly processed and served as negative control.

Sections from wild-type and TAP1 -/- mice brains were processed in the same solutions for immunohistochemistry using polyclonal antibodies raised in rabbit against the measles virus nucleocapsid protein [Norrby et al., 1982]. One series of sections was preincubated with 5% normal goat serum in PBS and 0.3% Triton X-100 for 30 min and then incubated in the primary antibody (diluted 1:10,000 in 2% normal goat serum/PBS/Triton X-100) overnight at room temperature. The sections were then exposed to biotinylated goat anti-rabbit IgG in 2% normal goat serum/PBS/Triton X-100 for 30 min, to avidin-biotinylated horseradish peroxidase complex (DAKO A/S, Glostrup, Denmark) for 30 min, and finally reacted for 15 min in Na-acetate buffer, pH 5.3, containing 0.02% 3-amino-9-ethylcarbazole and 0.01% H_2O_2. Each step was preceded by rinsing the sections in PBS twice for 10 min. The adjacent series of sections was stained with cresyl violet and used for histopathological and cytoarchitectonic studies.

RESULTS

Course of Disease

Wild-type or TAP1 -/- mice did not display obvious signs of disease until 6 d p.i. Starting from 7 d p.i., a few mice from each group were less reactive towards external stimuli. During the following days, these mice displayed brief episodes of hyperactivity and seizures (lasting 5–10 sec) in the form of forelimb-myoclonus accompanied by rearing. Six of the wild-type mice died between the 10^{th} and 13^{th} d p.i and one died at 18 d p.i., while 4 of the TAP1 -/- mice died between the 9^{th} and 13^{th} d p.i. and one at 19 d p.i. The remaining mice from both groups did not show apparent signs of disease until 28 d p.i., when they were sacrificed and sampled for histological examination (five mice from each group).

Time Course and Distribution of Virus Immunoreactivity

Measles-positive cells were not detected in the brains sampled from either wild-type or mutant mice in the 2^{nd} d p.i. When examined 4 and 7 d p.i., a few immunopositive cells and nerve fibers were seen around the needle track and bilaterally in the anterior olfactory nucleus (AON) in both wild-type and TAP1 -/- mice. Measles virus-immunopositive clusters of neurons were detected 10 d p.i. in wild-type mice: Immunostained neurons were found in the ipsilateral AON in three mice with signs of seizures; in addition, a few immunopositive neurons were observed in the ipsilateral piriform cortex and contralateral AON in one mouse, which was in a moribund state. In contrast, the spread of virus within the brains of TAP1 -/-mice sampled 10 d p.i. was markedly enhanced: numerous infected neurons were observed in the ipsilateral AON, piriform cortex, amygdaloid nuclei, and entorhinal cortex in two of these mice.

In the 14^{th} d p.i., immunoreactivity could not be detected in three of the four infected brains sampled from wild-type mice. However, in the fourth wild-type animal clusters of infected neurons were seen in the ipsilateral dorsal part of the AON, in the taenia tecta, as

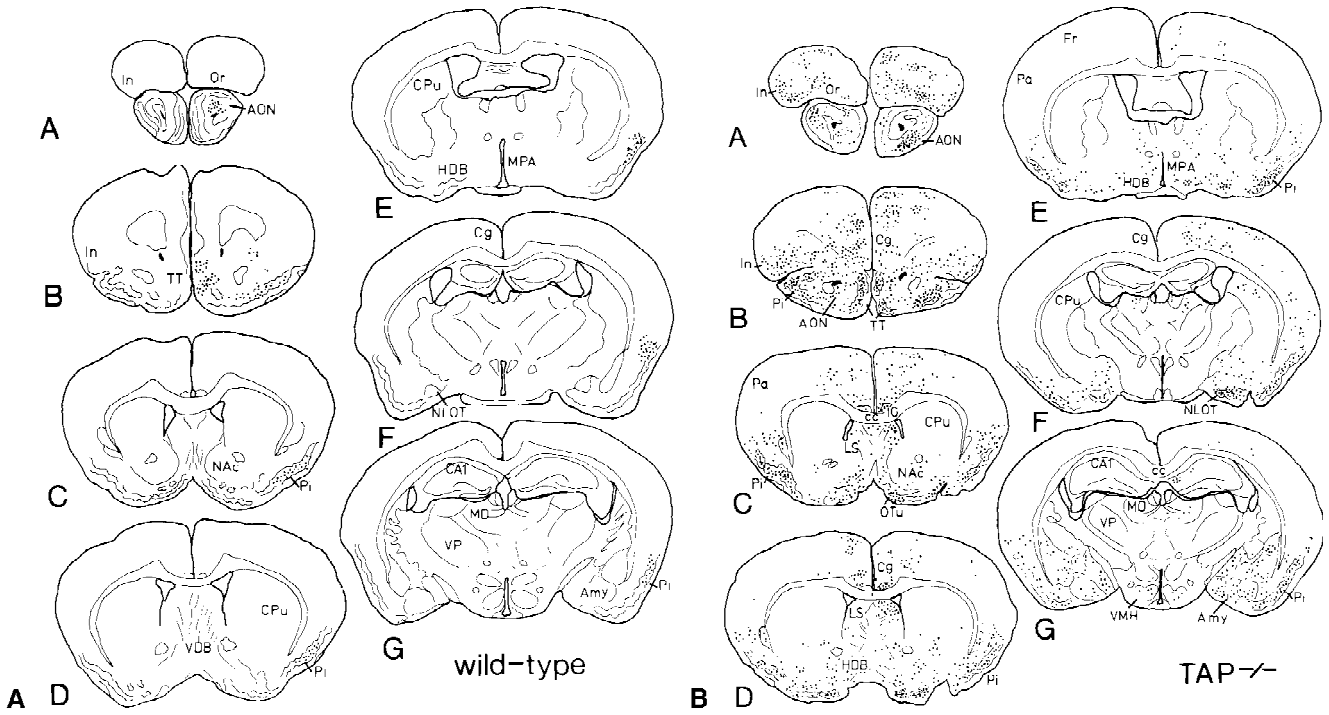

Fig. 1. Diagram of the distribution of immunopositive neurons in anteroposterior (A–G) sections through the brain of a wild-type (**A**) and a TAP1 -/- (**B**) mouse 14 d p.i. after injection of measles virus in the right olfactory bulb. Abbreviations: Amy, amygdaloid complex; AON, anterior olfactory nucleus; CA1, CA1 field of the hippocampus; cc, corpus callosum; Cg, cingulate cortex; Cpu, caudoputamen; Fr, frontal cortex; HDB, nucleus of the horizonatal limb of the diagonal band; IG, indusium griseum; In, insular cortex; LS, lateral septal nucleus; MD, mediodorsal thalamic nucleus; MPA, medial preoptic area; Nac, nucleus accumbens; NLOT, nucleus of the lateral olfactory tract; Or, orbital cortex; OTu, olfactory tubercle; Pa, parietal cortex; Pi, piriform cortex; TT, taenia tecta; VDB, nucleus of the vertical limb of the diagonal band; VMH, ventromedial hypothalamic nucleus; VP, ventroposterior thalamic nucleus.

well as throughout the anteroposterior extent of the piriform cortex, especially in its most dorsal portion, invading slightly the agranular insular and entorhinal cortices (Figs. 1A and 3A,C,E). On the other hand, 2 weeks after the infection the TAP1 -/- mice displayed a pronounced infection of the brain, and the distribution in one representative case is illustrated in Figure 1B. Overall, the immunopositive cell bodies prevailed on the side ipsilateral to the virus injection, but they were also numerous on the contralateral side of the brain. The infected neurons, in which the immunostaining extended along the dendritic arborizations, were grouped mainly in clusters (Fig. 5). Immunopositive neurons were found ipsilaterally in several portions of the AON, where large clusters were located in the posteroventral portions of the nucleus, whereas contralaterally a smaller number of immunopositive neurons was clustered dorsally in the AON (Fig. 2). Immunoreactive neurons densely filled the piriform cortex, throughout its extent (Figs. 3B,D,F and 4A,B), invading the overlying insular cortex and posteriorly the entorhinal cortex. In addition, clusters of immunostained cells were densest in the taenia tecta, olfactory tubercle, and nucleus of the lateral olfactory tract, and small-sized positive cells were packed in the indusium griseum. Immunostained neurons were also detected in other forebrain structures and, in particular, in the lateral septum as well as scattered throughout the nucleus of the horizontal limb of the diagonal band, and in several nuclei of the amygdaloid complex. Clusters of infected neurons were also detected in the orbital, insular, frontal, and cingulate cortices, and a few immunopositive neurons were observed in the CA1 field of the hippocampus. At diencephalic levels, a few immunostained cells were scattered in the medial preoptic area and in the ventromedial hypothalamus, as well as in the thalamus, where they were located in the parataenial nucleus and in the medial portion of the mediodorsal nucleus. In the brainstem, immunostained neurons were confined to the locus coeruleus (Fig. 4C,D) and dorsal raphe nuclei.

No immunopositive neurons were found in the brain of wild-type and TAP1 -/- animals sampled in the 28th d p.i.

Histopathological Features

In the second week of infection (10 and 14 d p.i.), measles virus-infected wild-type mice showed a marked lymphocyte infiltration in the leptomeninges, around blood vessels and in the parenchyma in infected areas of the brain. In contrast, lymphocyte infiltration was scarce in brains of the measles virus-infected TAP1 -/- mice. In heavily infected areas in brains from TAP1 -/- mice, tissue necrosis occurred with activation of rod-shaped microglial cells. Loss of pyramidal neurons in the CA1 and CA3 sectors of the hippocampus

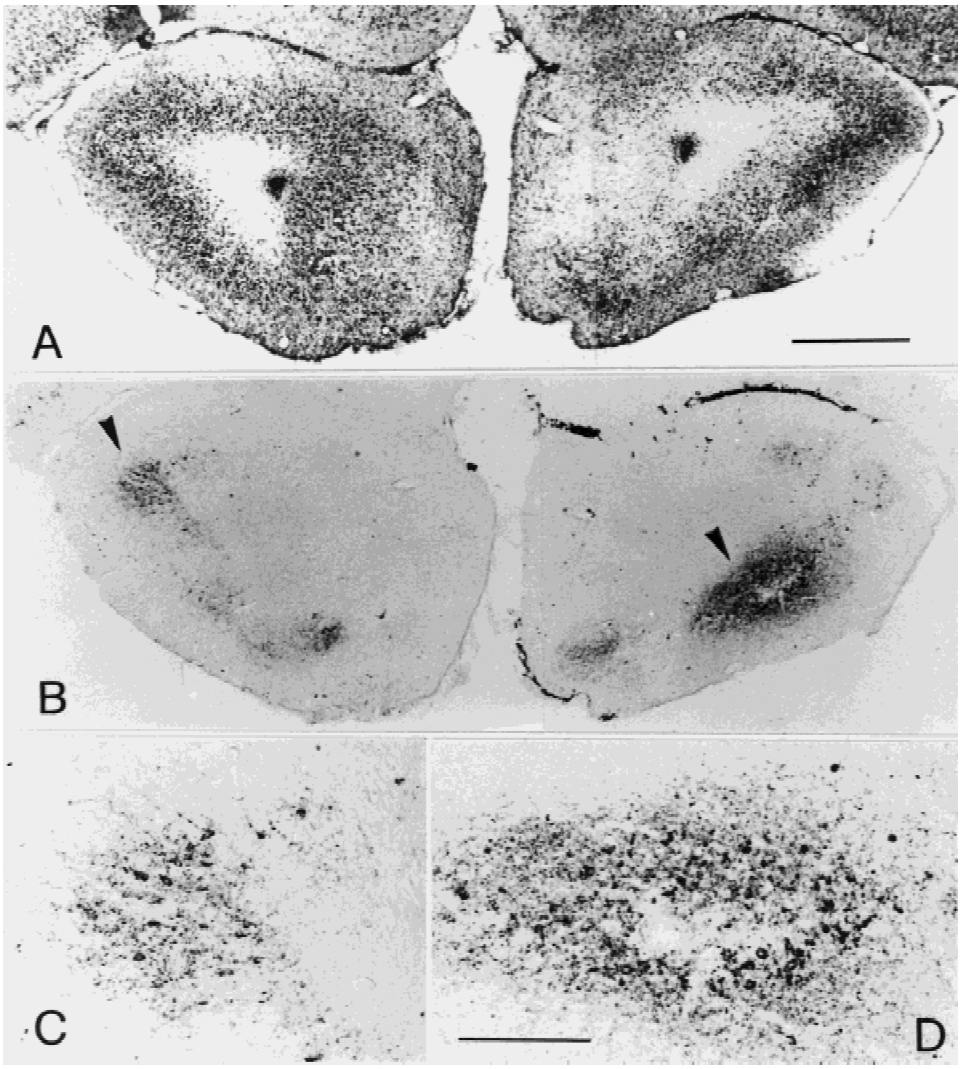

Fig. 2. Photomicrographs illustrating the AON in a TAP1 -/- mouse 14 d p.i. **A**: cresyl violet-stained section adjacent to the immunoreacted section shown in **B**. **C, D**: A higher magnification of the regions marked by arrowheads in B. Note the large cluster of infected neurons in the posteroventral portion of the AON on the right side (ipsilateral to the measles virus injection in the olfactory bulb) and a smaller, dorsal cluster on the contralateral side. Scale bars = 570 μm in A and B); 90 μm in C and D.

was evident bilaterally in infected brains of both wild-type and TAP1 -/- mice and occurred in mice, which clinically had seizures since the 10th d p.i.

DISCUSSION

Axonal Spread of Measles Virus

Previous experimental studies on measles virus infection of the brain have employed direct inoculations into the cerebral hemispheres [Neighbour et al., 1978; Rammohan et al., 1980; Percy and Coulter-Mackie, 1982; Chan, 1985; Löve et al., 1986; Andersson et al., 1990, Eastman et al., 1993], and it has been suggested that measles virus spreads into the brain by infecting endothelial cells during secondary viremia [Esolen et al., 1995]. Inflammatory changes in the brain have, however, been reported following intranasal virus instillation in hamsters, thus suggesting that the nasal cavity can provide an access to the brain infection [Zlotnik and Grant, 1976]. The present data provide the first direct evidence that measles virus is able to spread within the brain along olfactory routes and reach limbic structures. Our findings show that infected neurons were distributed selectively in the structures connected through axonal pathways with the olfactory bulb. Thus, infected neurons were detected in areas connected reciprocally with the olfactory bulbs, such as the ipsi- and contralateral AON, piriform and entorhinal cortices, and amygdaloid and septal nuclei [Carson, 1983; Shipley and Adamek, 1984]. In addition, measles virus was found in neurons of areas recipient of olfactory bulb efferents, such as the indusium griseum [Wyss and Sripanidkulchai, 1983], and in areas that give origin to olfactory bulb afferents, such as the nucleus of the horizontal limb of the diagonal band, the locus coeruleus and the dorsal

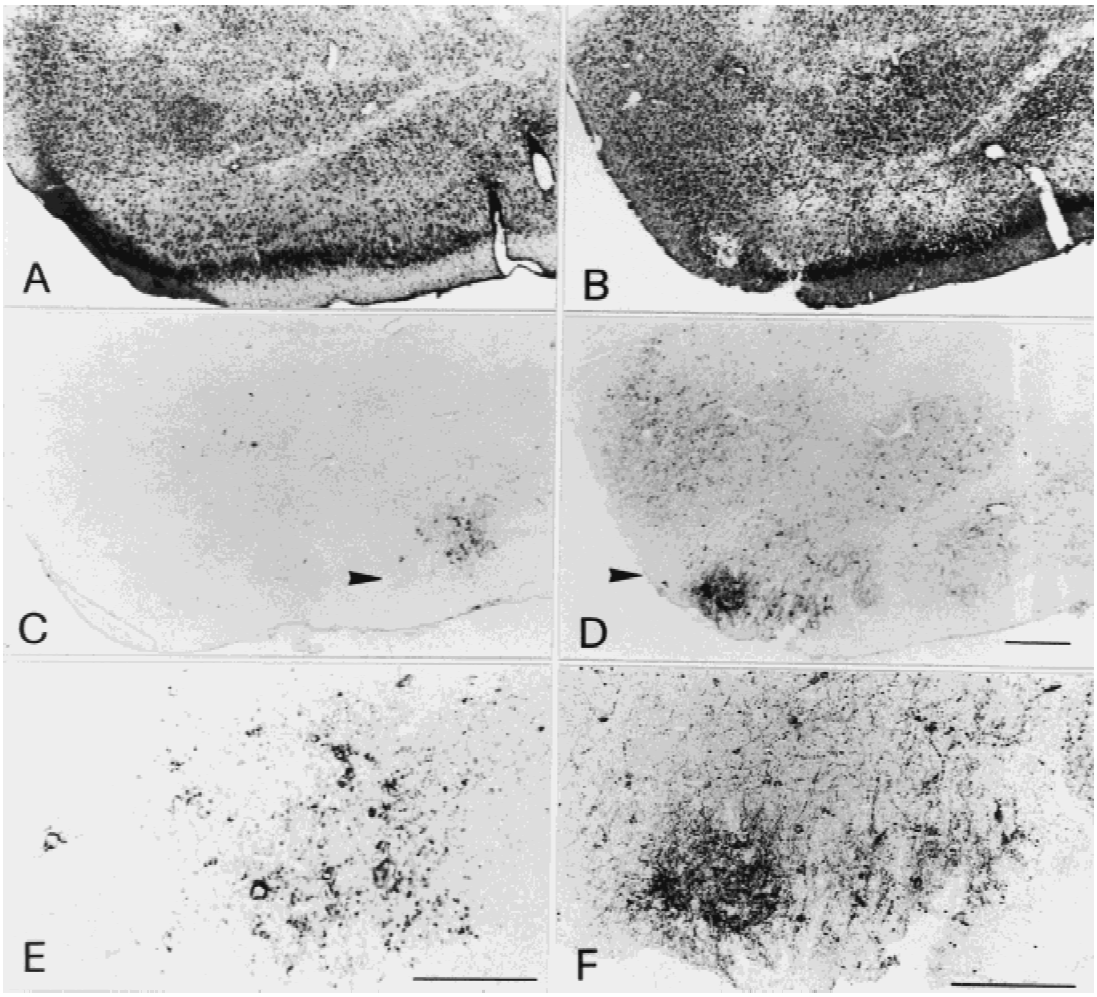

Fig. 3. Photomicrographs of the piriform cortex and amygdala in a wild-type (**A,C,E**) and a TAP1 -/- (**B,D,F**) mouse ipsilateral to the measles virus injection, 14 d p.i. A,B: Cresyl violet-stained sections adjacent to the immunoreacted sections shown in C and D. E,F: A higher magnification of the indicated areas. Note that in the piriform cortex of the wild-type mouse the infection is more restricted than in the TAP1 -/- mouse, in which the infection also involved the amygdaloid complex. Scale bars = 320 μm in A–C,D, 150 μm in E, and 230 μm in F.

raphe [Shipley and Adamek, 1984]. These data therefore indicate that measles virus can be transported both anterogradely and retrogradely in axons. Furthermore, the pattern of virus propagation into the limbic structures and its temporal schedule indicate that the virus can pass transneuronally to the next order of neurons from infected cells. In a recent study on human brain material from patients with subacute sclerosing panencephalites a cephalo-caudal, transneuronal spread of the measles virus in the brain was suggested [Allen et al., 1996]. The closely related paramyxovirus, canine distemper virus, shows after intracranial injection in a mouse model an antigen distribution suggestive of a virus spread in axons within the brain [Bernard et al., 1993].

The Role of Functional MHC Class I-Mediated Antigen-Presenting Pathway in Restricting Viral Spread in the Brain

The extent and pattern of viral spread is related both to the type or strain of virus and to factors that permit or restrict virus replication in the host. These factors include expression of virus receptors on different cell types and the cellular metabolic machinary that influences virus replication, as well as the induction of antiviral factors, such as interferons, and the humoral and cell-mediated immune response. The present findings provide evidence that the dissemination of measles virus in the brain is enhanced in the TAP1 -/- mice. A crucial role for an intact TAP complex in the presentation of viral antigens to CTL has been extensively demonstrated in cell lines in vitro [Townsend et al., 1989; for review see, Yewdell and Bennink, 1992]. However, surprisingly little documentation exists with respect to the role of a functional TAP complex in the presentation of viral antigens in vivo. To our knowledge, the role of the immune response to a virus has been addressed in vivo using TAP1 -/- mice only in one previous study, in which TAP was studied in relation to the presentation of an immunodominant lymphocytic choriomeningitis virus-derived CTL epitope localized in the signal sequence of a virus protein [Hombach et

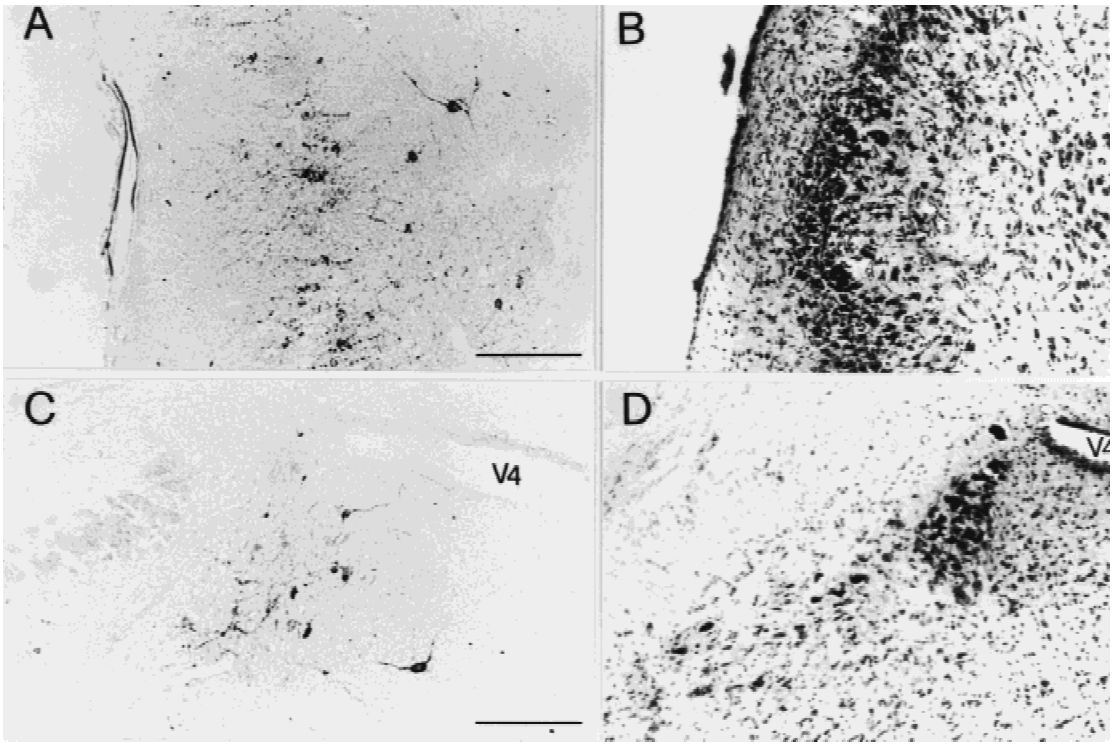

Fig. 4. Photomicrographs illustrating the piriform cortex contralateral to the measles virus injection (**A,B**) and the locus coeruleus (**C,D**) on the ipsilateral side in a TAP1 -/-mouse 14 d p.i. B,D: Cresyl violet-stained sections adjacent to the immunoreacted ones (A and C). Scale bars = 150 μm. V4, fourth ventricle.

al., 1995]. In the present investigation, the spread of measles virus and the scarcity of lymphocyte infiltration in TAP1 -/- mice point to the importance of the ability to process and present antigen on MHC class I to cells of the immune defense, and, in particular, of a functional TAP complex in the control and clearing of virus brain infections in vivo.

Under physiological conditions, MHC class I molecules are not expressed, or are expressed at very low levels, on the cells of the central nervous system. Therefore, the present observation that TAP1 plays a role during an infection in the brain may seem paradoxical. However, MHC class I molecules can be upregulated in neurons by a variety of stimuli [e.g., Maehlen et al., 1988; Neumann et al., 1995], which include measles virus infections [Maehlen et al., 1989; Gogate et al., 1991]. This indicates that neurons can be a target of CTL, which therefore may play a role in disorders hallmarked by nerve cell degeneration and death. This is consistent with an observation of MHC class I expression in neurons in brains from patients with subacute sclerosing encephalitis [Gogate et al., 1996]. In this study only a small proportion of the measles virus-infected neurons expressed MHC class I antigen, which may suggest that such cells are cleared rapidly by the CTL.

Measles Virus-Induced Seizures

In the present study, bilateral loss of pyramidal cells in the hippocampus was detected in both wild-type and TAP1 -/- mice. Such neuronal loss is probably secondary to the limbic seizures observed in the infected mice, since very few, if any, infected neurons were detected in the hippocampus. We have previously reported that systemic application of an NMDA glutamate receptor antagonist can prevent hippocampal neurodegeneration following intracerebral inoculations of measles virus in BALB/c mice [Andersson et al., 1990], which indicates the involvement of excitatory amino acids in the pathogenesis of the virus-induced neuronal changes. In addition, the occurrence of measles virus-induced hippocampal necrosis is preceded by an increase in the activity of enzymes synthesizing quinolinic acid, which is an endogenous NMDA receptor agonist [Eastman et al., 1994]. In the present study, seizures and hippocampal necrosis occurred also in measles virus-infected wild-type mice, in which the infection did not spread beyond the piriform cortex. This is of interest because this region may have a low convulsive threshold [Haberly and Sutula, 1992], and experimental studies have indicated that propagated seizures may be evoked from its rostral part [for review, see Gale, 1995].

In relation to human measles infections, of particular interest in the present context are reports of "subacute measles encephalitis" in young immunocompromised patients, 1–7 months after a measles virus infection. Seizures and mental alterations represent the most common clinical signs of the afflicted individuals. The infection is usually lethal and most of the few sur-

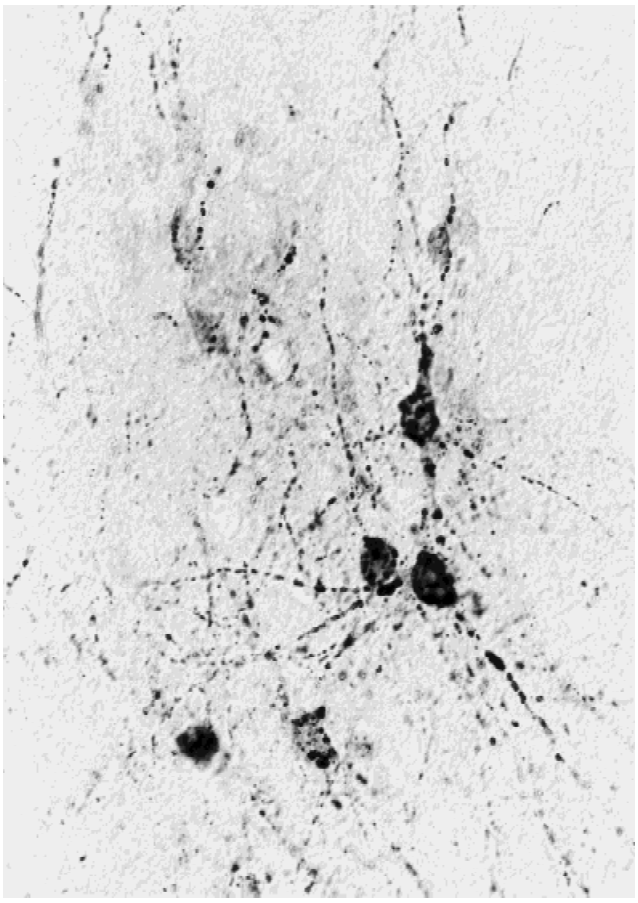

Fig. 5. Group of infected neurons in the entorhinal cortex of a TAP1 - /- mouse 14 d p.i.; note the extent of the immunostaining into the dendritic branches.

viving patients, who had received antiviral treatment, have displayed severe psychomotor disturbances on follow-up examinations [for review, see Hughes et al., 1993; Mustafa et al., 1993].

Variations in Targeting of Different Viruses to Limbic and Monoaminergic Neurons

The topographical distribution of a viral infection in the brain is not only a result of the capacity of the virus to propagate in anterograde and/or retrograde directions within axons, but also of the susceptibility of different nerve cell populations to the virus attack. The presence of virus receptors on a cell is one such determining factor. Recently, a number of studies have implied that human CD46, or membrane cofactor protein, may serve as the receptor for measles virus [Buchholtz et al., 1996; Manchester et al., 1994]. Since this molecule is not present in rodents, it is apparent that alternative receptors are used by the hamster-adapted neurotropic strain of measles virus used in the present study.

Due to the different determining factors for virus targeting in the brain, a variety of viruses that can spread along olfactory pathways display a selectivity in their attack on brain regions. For instance, dorsal raphe neurons are infected after intranasal application of vesicular stomatitis virus and of wild-type, but not avirulent, strain of rabies [Lundh et al., 1988; Lafay et al., 1991; Hunneycutt et al., 1994]. Neurons of the locus coeruleus are infected by herpes simplex virus, but not by mouse hepatitis virus [Barnett et al., 1993]. Borna disease virus and Theiler's virus can also spread along olfactory pathways, but the pattern of their ensuing distribution in the brain differs to a certain extent [Carbone et al., 1987; Morales et al., 1988; Wada and Fujinami, 1993].

The present findings support the concept that a broad spectrum of neurochemical and behavioral alterations can represent the consequence of the propagation of different viruses within brain regions. Human neuropsychiatric and neurodegenerative diseases of unknown etiology may be related to dysfunctions in these structures of the brain. Therefore, the study of host factors, genetic, or inherent in the immune defense, that govern the propensity of infectious agents to invade the brain through a natural port of entry, can open new avenues to understand pathogenetic mechanisms behind such disorders.

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